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I,
Alain CATHERINE
c/o Cabinet HARLE & PHELIP
7 rue de Madrid
75008 PARIS
France

hereby declare that I am conversant with the French and the English languages and I certify that to the best of my knowledge and belief the following is a true and correct English translation of the specification contained in International patent application n° PCT/FR2004/050308 filed on July 2, 2004 in the names of :

1) INSERM (Institut National de la Santé et de la Recherche Médicale)

2) CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE

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Alain CATHERINE

FIELD OF THE INVENTION

The present invention relates to the field of immune booster compounds, that is to say compounds that are able to induce an increase in the immune response against an antigen, so as to improve the immune response stimulation efficiency by means of an immunogenic composition, or to improve the prophylactic or therapeutic efficiency of a vaccinal composition.

STATE OF THE ART

Generally speaking, compounds or compositions having an immune booster function are useful for improving the stimulation conditions of an immune response against antigens. Usually, immune booster compounds or compositions are used to increase the amount of antibodies that are produced against a specific antigen, or to increase the amount of produced effector T-cells, whether the so called "Helper T-cells" (T-helper) or the cytotoxic T-cells.

In general, associating an antigen with an immune booster compound or composition not only results in an increase of the immune response level due to a higher production of antigen specific antibodies or effector T-cell, but makes it also possible to reduce the antigen amount comprised in an immunogenic or vaccinal composition, and, eventually, to reduce the injection frequency of said immunogenic or vaccinal composition.

An immune booster and the interesting antigen have especially to be associated with each other where the immunogenic properties of such interesting antigen, when administrated without any adjuvant, are not sufficient to stimulate an efficient immune response considering the immunization objectives aimed at.

Depending on their nature, immune booster compounds or compositions induce a better immune response against an interesting antigen according to distinct ways. Some immune booster act on the immune system by inducing a more efficient antibody production against the interesting antigen, for example by activating macrophages, dendritic cells, B-cells and T-cells, or by improving the conditions in which the interesting antigen is presented to the various immunocompetent-cells.

Immune booster compounds or compositions may increase the immune response by extending the release duration of the interesting antigen, by increasing the antigen amount absorbed by the antigen presenting cells, by positively regulating the so called antigen processing effected by the cells, by stimulating the cytokine release, by stimulating the so called isotypic switching and the B-cell maturation and/or by eliminating the immunosuppressive cells.

Amongst the various immune booster compounds or compositions, those that enable the interesting antigen(s) to better present to the immunocompetent-cells, and more especially to the so called helper T-cells or lymphocytes (or $CD4^+$ cells) or T-cytotoxic lymphocytes (CTL or $CD8^+$ cells), are very interesting to prepare efficient immunogenic compositions or vaccinal compositions. Immune system cells processing antigens by fragmenting them into peptides, then by presenting these peptides, in combination with class I or class II major histocompatibility complex (MHC) molecules, are essentially macrophages and dendritic cells. Dendritic cells are able to process antigens, then to present peptides resulting from antigen processing to naive T-cells. Dendritic cells activate more efficiently T-cells than any other antigen-presenting cell. Moreover they are needed for the initial activation of the naive T-cells, either *in vitro* or *in vivo*.

Dendritic cells are generally present in the body system in sites that are exposed to foreign antigens, such as skin, liver, intestine, blood and lymphoid tissue. On the whole, dendritic cells are classified depending on the maturation stages, whether they are mature or immature. Mature dendritic cells are able via endocytosis to collect antigens and to process them efficiently, and also to express high levels of costimulating molecules, such as CD40, CD80 and CD86 molecules, as well HLA-DR major histocompatibility complex molecules. Moreover, mature dendritic cells express CD83 marker and secrete high levels of various cytokines and chemokines functioning as helpers in activating T-cells.

In addition to their role in activating naive T-cells, mature dendritic cells also may influence Th1/Th2 immune response balance. Many studies demonstrated that dendritic cells preferably activate Th1 type responses, probably thanks to secretion of IL-12 by the activated dendritic cells.

(Macatonia and al., 1995, J. Immunol., vol. 154:5071; Hilkens and al., 1997, blood, vol. 90:1920). Nevertheless, many studies demonstrated that dendritic cells may indifferently induce generation of Th1 or Th2 cell clones. (Roth and al., 1996, Scand. J. Immunol., vol. 43:646).

The dendritic cell significant role in processing the antigen and in activating T-cells mobilized much attention as how to use or activate dendritic cells in immunotherapy. Mature dendritic cell activation or production is especially interesting in the field of vaccines and cancer immunotherapy.

It has been shown according to the state of the art that nucleic acids containing CpG type oligonucleotide sequences, were able to induce dendritic cell maturation. Recently, autologous dendritic cells have also been used, that were obtained from patients suffering from cancer in the context of cancer immunotherapy, as described especially in the PCT application publication n° WO 98/23728. It has also been shown that ring system comprising chemical compounds selected from the group consisting in ring systems such as imidazoquinoline, imidazopyridine, cycloalkyl-imidazopyridine, imidazonaphthyridine or imidazotetrahydronaphthyridine, were able to induce *in vitro* immature dendritic cell maturation, such as described in US patent N°6,558,951.

There is a need in the state of the art for new immune booster compounds able to activate dendritic cells and to induce their maturation, so as to obtain a high level immune response against an interesting antigen, whether by producing specific antibodies directed against such interesting antigen in a great amount, or by stimulating helper T-cells or cytotoxic T-cell proliferation that are specific to such interesting antigen.

SUMMARY OF THE INVENTION

According to the present invention, there has been characterized a peptide type new immune booster compounds family, that activate dendritic cells and induces immature dendritic cell maturation.

Immune booster compounds according to the present invention are derived from the "knob" domain of the fiber protein of an adenovirus capsid.

More specifically, it is an object of the invention to provide an immune booster compound comprising:

- a polypeptide (i) comprising a 30 amino acid-long amino acid sequence contained in the "knob" domain of the fiber protein of an adenovirus capside, said amino acid sequence comprising the amino acid chaining forming double β-sheet structure referred to as "EF" contained in said "knob" domain; or
- a peptide (ii) analogous to said polypeptide (i), the amino acid sequence of which comprises, as compared to said polypeptide (i) sequence, at least one substitution or at least one deletion of an amino acid, said analogous peptide retaining said double β-sheet structure referred to as "EF".

The invention also relates to an immune booster composition comprising a booster compound such as defined hereabove, in combination with at least one physiologically compatible excipient.

It also relates to an immunogenic composition as well as to a vaccinal composition comprising a booster compound such as defined hereabove, in combination with at least one interesting antigen.

The invention further relates to a method for the *in vitro* maturation of human or animal immature dendritic cells, characterized in that it comprises following steps consisting in:

a) *in vitro* culturing in a suitable culture medium a cell population enriched with human or animal immature dendritic cells; and

b) incubating the cells cultured in step a) with a booster compound or a booster composition such as defined hereabove, for a time sufficient to induce dendritic cell maturation.

The invention further relates to a cell population enriched with mature dendritic cells, that may be obtained by means of the hereabove maturation method.

It is another object of the present invention to provide an immune booster composition, characterized in that it comprises a mature dendritic cell population obtained by means of the hereabove maturation method.

The invention also relates to a method for stimulating *in vitro* antigen-specific T-cells, characterized in that it comprises following steps consisting in:

a) preparing a mature dendritic cell-enriched cell population by means of the hereabove maturation method;

b) contacting the mature dendritic cell-enriched cell population obtained in step a) with a T-cell-enriched cell population coming from the same individual, either a human or an animal.

DESCRIPTION OF THE FIGURES

Figure **1** shows schematically the structure of the fiber protein in serotype 5 adenovirus virions, respectively wild (figure 1A) and virus without the "knob" domain (figure 1B).

The adenovirus fiber protein comprises three structural domains, beginning at the N-terminal end up to the C-terminal end: The "tail" which is not covalently linked to the part "penton base"; the "shaft" and the "knob" domain binding to the CAR receptor, which acts in binding the adenovirus to the permissive cells. Ad5 serotype wild adenovirus has "fibers" comprising a long "shaft" having 22 repeating β-sheet units. With the mutant vector without the "knob" domain used according to the present invention (AdE1° Δ knob, see figure 4C), the fiber protein "shaft" is shorter, having seven repeating β-sheet units and terminating with a trimerization unit followed by an "opal" stop codon.

Figure **2** shows the effect of adenovirus capsid components effect on dendritic cell phenotype.

Dendritic cells were cultured in the culture medium alone (NS; non stimulated dendritic cells), or in presence of Ad5 adenovirus purified proteins (1 μg x 10^6 cells), or LPS (1 μg/ml); then they were characterized for their CD11c marker expression and for their expression of the dendritic cell-specific maturation markers.

Figure **2A** shows dendritic cells cultured with Hx and Pb (penton base bound to the fiber) isolated from Ad5 adenovirus-infected HeLa cells.

Figure **2B** shows dendritic cells cultured with Hx, Pb or Fi recombinant proteins purified from baculovirus-infected Sf9 cells, or with a Pn protein (penton) purified from Ad5 adenovirus-infected HeLa cells.

Fluorescence intensity mean values (MFI) appear on different panels of figure 2A and are given on ordinates in figure 1B. Indicated results represent three independent assays.

Figure **3** shows Ad5 adenovirus knob protein dose-related effect on dendritic cell maturation.

Dendritic cells were incubated without "non stimulated dendritic cells, NS " or in presence of 1μg Fi protein or knob domain protein (figure 3A), wherein dendritic cells were incubated with defined amounts of Fi and Hx proteins (1 μg, respectively) and at varying knob protein concentrations (ranging from 0.5 to 0.02 μg) (figures 3B, 3C).

Dendritic cells and culture supernatants were collected and tested as indicated hereafter. For figure 1A, dendritic cells were characterized for CD11c marker and maturation marker expression. CD11c+ cell percents and maturation marker immunofluorescence mean values (MFI) are indicated.

In figure **3B**, dendritic cell increasing numbers were used as stimulating cells, with allogenic purified CD4+ T-cells. Cell proliferation values, evaluated by incorporating ^{3}H-thymidine, are expressed in cpm (mean based on three assays; m ± SD).

In figure **3C**, IL12 p70 and TNFα amounts have been determined by means of the ELISA method from dendritic cell culture supernatants, and results are given in nanograms x 10^{6} cells. Data result from at least three independent assays and standard deviation values (SD), ranging around 15% of mean values, are not given.

Figure **4** demonstrates the direct interaction between knob domain protein and dendritic cells, and the need for the knob domain in Ad5 adenovirus-induced dendritic cell maturation.

For figure **4A** dendritic cells purified by means of anti-CD11c antibody-coated magnetic beads were stimulated with 0.5 μg of knob domain protein; non stimulated purified dendritic cells (NS) were used as controls. CD11c+ cell percents and maturation marker immunofluorescence mean values (MFI) are given in abscissa.

For figure **4B**, dendritic cells were stimulated with 1 μg Fi protein (complete fiber protein), then incubated with mouse monoclonal antibodies anti-tail domain of the fiber protein (anti-Fi-tail). Percentage of fluorescent cells having bound the fiber protein has been determined after deduction of anti-Fi monoclonal antibody non-specific binding onto non stimulated dendritic cells (NS-DC).

For figure **4C**, dendritic cells were not stimulated (NS), or were stimulated with, respectively, either Ad5E1 virus carrying a wild-type fiber

protein (WT-Fi-Carrying Ad5E1) or with Ad5E1°Δ knob vector without the knob domain, i.e. $1x10^4$ particles/cell. Histograms give immuno-fluorescence mean values (MFI) for CD11c+ cell maturation markers. Indicated results represent two independent assays.

Figure **5** shows the CAR receptor absence of expression on dendritic cells and the dendritic cell low permissivity towards the serotype 5 adenovirus-induced infection.

Immature dendritic cells isolated before any stimulation were incubated with an anti-CAR monoclonal antibody-containing ascite liquid (line in bold), or with an unrelated ascite liquid (line in grey).

Dendritic cells were assayed by means of flow cytometry (FACS). The HeLa and CHO cells were used respectively as positive and negative controls.

Figure **6** is a mapping of the knob domain region for the fiber involved in dendritic cell maturation.

Figure **6A** diagrammatically illustrates the conformation structure of the Ad5 adenovirus fiber protein knob domain (Xia and al., 1994).

This diagram shows the various β-sheet structured regions from A to J, as well as respective binding loops AB, CD, DG, GH, HI and IJ.

Amino acid numbering is indicated on top line, starting from N-terminal methionine residue of Ad5 adenovirus complete fiber protein. The regions of fiber protein knob domain interacting with CAR receptor are illustrated as filled black boxes on the bottom line of the figure.

Figure **6B** linearly illustrates the various deleting mutants of the Ad5 adenovirus fiber protein knob domain. Knob domain sequence is represented as filled boxes; deleted regions are represented as fine lines.

For figure **6C**, non stimulated dendritic cells (NS), extract-stimulated dendritic cells with no knob domain (MS; "mock-stimulated "), or wild-type knob domain protein-stimulated dendritic cells ("knob WT ") or fiber protein knob domain deleting mutant-stimulated dendritic cells were used, respectively, as stimulating cells towards allogenic, purified CD4+ T-cells. Indicated results are means of three separated assays. It has to be noted that amongst the given results only 0.5 µg of knob domain protein were used, as compared to 2 µg of mutant protein.

Figure **7** shows that Ad5 adenovirus fiber protein knob domain-stimulated dendritic cells stimulate *in vivo* CD8+ T-cells specific to LCMV glycoprotein-derived GP33 antigenic peptide.

Figure **7A** shows donor cell rejection in dendritic cell-vaccinated recipient mice. CFSE-labeled (5-6-carboxyfluorescein diacetate succinimidyl ester) B6 mice splenocytes were charged with GP33 peptide, then treated splenocytes were transfused into recipient B6 mices which had been previously immunized with, respectively, (i) fiber protein knob domain-stimulated dendritic cells (respectively 2.5×10^5, 9×10^4 and 3×10^4 cells), charged with GP33 or (ii) with dendritic cells charged with GP33 (2.5×10^5 cells) stimulated with Pb or Hx, or not stimulated (NS), or (iii) with dendritic cells charged with NP366 (2.5×10^5) stimulated with the fiber protein knob domain, or (iv) with the GP33 peptide in emulsion in Freund's incomplete adjuvant (IFA).

Rejection level, as expressed in percent, was evaluated in blood and in the spleen at different moments following the adoptive cell transfer.

Figure **7B** shows *ex vivo* IFN-γ secretion in the same mice than in figure 7A. Spleen cells and freshly isolated peripheral blood lymphocytes (PBL) were incubated for 20 hours with GP33 peptide; the number of GP33-specific cells secreting IFN-γ was evaluated by means of an Elispot IFN-γ assay; results are expressed in SFC ("spot forming colony")/10^5 CD8+ T-cells (triplicate test mean ± SD). Each spot corresponds to an IFN-γ-secreting cell.

Figure **7C** shows the effector cell cytolytic activity that are derived from mices vaccinated with dendritic cell (3×10^4 cells) and with non stimulated dendritic cells or stimulated with Ad5 adenovirus fiber protein knob domain. After a 4 day-long *in vitro* stimulation with the GP33 peptide, spleen cells were incubated with ^{51}Cr-labelled EL4 target cells, then charged with the GP33 peptide or the NP366 peptide; in a chromium release test, only the GP33-specific activated ones will lyze the targets (^{51} Cr).

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, it has been shown that the knob domain of the capsid fiber protein of an adenovirus may directly interact

with immature dendritic cells and cause their activation and their maturation into mature dendritic cells.

Adenoviruses come as icosaedric particles from 70 to 100 nanometers diameter, depending on the serotype. Virus capsid comprises two main components, respectively, (i) the hexon (Hx) that forms the sides and the penton (Pn) placed on the 12 vertices.

Penton base is associated to the fiber protein, which is a spicular projection coming from penton base, made of a IV polypeptide trimer. The fiber is a protein having from 581 to 587 amino acid residues for the long fiber species adenoviruses, such as C and A species, and from 319 to 325 amino acid residues for most shorter fibers such as 3 and 7 serotype adenoviruses, belonging to the B species.

Fibers all comprise three separate structural domains, respectively the tail, the shaft and the knob. The tail fit into the penton-base. The shaft is made of a periodical repeating unit comprising about fifteen residues each representing a β-sheet structure. The number of repetitions of the single unit defines the shaft length which may vary depending on fibers of different adenovirus species. There is at the C-terminal end of the fiber a rounded zone, the head, that is made of a 180-200 amino acid residue-long polypeptide sequence trimer. As illustrated in figure 6A, each knob monomer associates a backbone comprising eight antiparallel β-sheet bound to each other with loops, the conformation of which substantially varies according to the serotypes.

Some authors had demonstrated that human adenovirus was able to mature human and murine dendritic cells *in vitro* (Rea and al., 99; Hirshowitz and al., 2000; Morelli and al., 2000; Rouard and al., 2000). Nevertheless, these fortuitous observations about the adenoviral vector activity on the material and on dendritic cells did not permit to identify the pathway to activation and to maturation of dendritic cells. So, these previous results did not permit to objectively determine which dendritic cell molecular activation and maturation mechanism was induced by the adenovirus or, a fortiori, which adenovirus component or component combination might be the causative agent(s) for that, especially amongst the twelve main polypeptides comprising the viral particle.

It has now been demonstrated according to the present invention that a polypeptide comprising a fragment belonging to the knob domain of the fiber protein of an adenovirus, said fragment comprising the amino acid sequence forming the antiparallel double β-sheet structure referred to as "EF", induces immature dendritic cell activation and maturation.

More precisely, it has been demonstrated according to the present invention that a peptide fragment of the adenovirus fiber protein knob domain comprising the amino acid sequence delimitating the antiparallel β-sheet EF and deleted in other peptide regions of the knob domain, such as for example the antiparallel β-sheet "HI", has immature dendritic cell activation and maturation properties similar to those observed for a polypeptide comprising the fiber protein complete knob domain sequence of said adenovirus.

On the other hand, it has also been shown that a polypeptide comprising the fiber protein knob domain and the deletion of the amino acid sequence forming the EF antiparallel β-sheet structure lost the immature dendritic cell activation and maturation properties of the complete knob domain. Even more precisely, it has been demonstrated that a polypeptide comprising the knob domain and the deletion of two amino acids in the F-region of the the EF antiparallel β-sheet does not have immature dendritic cell activation and maturation properties observed with a polypeptide comprising the knob domain complete amino acid sequence.

So, the results obtained by the applicant show that a polypeptide comprising a peptide fragment belonging to the knob domain of the fiber protein of an adenovirus, and comprising the amino acid sequence forming EF antiparallel double β-sheet structure, said polypeptide having no amino acid sequence forming other β-sheet structures contained in the knob domain, can induce immature dendritic cell activation and maturation.

The results obtained by the applicant show that immature dendritic cell activation and maturation properties of the knob domain of the fiber protein of an adenovirus are carried by a small peptide region of said knob domain, the amino acid regions forming EF double β-sheet structure.

The fiber protein knob domain of all adenoviruses has a common structure made of successive β-sheets that are bound to each other by

peptide loops, similar to those of the knob domain of serotype 5 adenovirus (Ad5) shown in figure 6A.

The applicant also demonstrated that, at least as regards the immature dendritic cell activation and maturation properties, this knob domain structure identity for all the adenoviruses also involves a function identity. So, it has been demonstrated according to the present invention that an adenoviral vector comprising chimeric fiber proteins comprising a tail and a shaft from a serotype 5 adenovirus and a knob domain from a serotype 3 adenovirus is also able to induce immature dendritic cell activation and maturation.

The hereabove results made it possible for the applicants to define a new family of immune booster compounds, derived from the knob domain of the fiber protein of an adenovirus, such new family of immune booster compounds representing a first object of the invention.

It is an object of the present invention to provide an immune booster compound comprising:

- a polypeptide (i) having a 30 amino acid-long amino acid sequence contained in the "knob" domain of the "fiber" protein of an adenovirus capsid, said amino acid sequence comprising the amino acid chaining forming double β-sheet structure referred to as "EF" contained in said "knob" domain; or
- a peptide (ii) analogous to said polypeptide (i), the amino acid sequence of which comprises, as compared to said polypeptide (i) sequence, at least one amino acid substitution or at least one amino acid deletion, said analogous peptide retaining said double β-sheet structure referred to as "EF".

It has been demonstrated in the examples that a booster compound according to the hereabove definition induces immature dendritic cell maturation. More particularly, an booster compound such as hereabove induces the expression by dendritic cells of MHC class I and class II molecules, as well as mature dendritic cell-specific markers, such as CD40, CD80 and CD86 markers. Booster compound-stimulated dendritic cells such as defined hereabove induce allogenic CD4+ T-lymphocyte proliferation in mixed lymphocyte reaction assays (MLR) and also induce IL-12 and TNFα secretion in a dose-related manner. A booster compound

according to the present invention directly acts on immature dendritic cells without binding on CAR receptor.

For booster compounds of the invention comprising a polypeptide (i), said polypeptide (i) has an amino acid sequence long of at least 30-amino acid since polypeptide (i) in all cases comprises a 30 amino acid-long amino acid sequence containing the amino acid sequence forming the EF β-sheet of the knob domain of the fiber protein of an adenovirus, which carries the immature dendritic cell maturation function.

For immune booster compounds comprising a peptide (ii) analogous to polypeptide (i), said analogous peptide (ii) has a structure very similar to that of polypeptide (i) and retains the basic structural characteristic i.e. comprising an amino acid sequence forming EF antiparallel double β-sheet structure.

In an analogous peptide (ii), the amino acid sequence of the EF β-sheet may comprise, as compared to the EF β-sheet amino acid sequence of adenovirus fiber protein knob domain from which it was derived, one or more amino acid substitution(s). Nevertheless, the amino acid substitution(s) in the EF β-sheet sequence are such that they do not modify said β-sheet structure.

However, most preferably, the amino acid sequence forming the EF β-sheet of an analogous peptide (ii) is the same as the amino acid sequence of parent polypeptide (i) EF β-sheet.

According to a preferred aspect of a booster compound according to the present invention, the amino acid chaining forming EF double β-sheet structure is localized approximately in the middle of the amino acid sequence of said polypeptide.

For example, EF β-sheet amino acid sequence of the serotype 5 adenovirus fiber protein knob domain is 8-amino acid long. As illustrated in figure 6A, this amino acid sequence begins with amino acid residue at position 479 and ends with amino acid residue at position 486 of the serotype 5 adenovirus complete fiber protein. For a booster compound comprising a polypeptide (i) with the minimum length of 30 amino acids, the 8 amino acid-long EF β-sheet amino acid sequence, is preceded at the N-terminal end by a 11 amino acid-long sequence corresponding to part of

the DE loop and is followed, at the C-terminal end, by a 11 amino acid-long sequence comprising part of the FG loop.

The EF β-sheet amino acid sequence comprised in a booster polypeptide (i) according to the present invention is localized "approximately" in the middle of the amino acid sequence of the polypeptide (i) where sequences localized at the N-terminal side and at the C-terminal side of the EF β-sheet sequence, respectively, have not the same length. For example, the length of sequences localized at the N-terminal side and at the C-terminal side of the EF β-sheet amino acid sequence, respectively, may have lengths varying in a proportion of up to 20 amino acids, compared to each other.

According to another preferred aspect of a booster polypeptide (i) according to the present invention, said polypeptide (i) comprises, from the N-terminal end towards the C-terminal end, the amino acid sequences of the DE loop, the EF β-sheet and the FG loop.

As an illustration, for such a polypeptide (i) type derived from adenovirus serotype 5 fiber protein, said booster compound comprises the polypeptide, the amino acid sequence of which begins with amino acid residue at position 463 and ends with amino acid residue at position 515 of the complete fiber protein sequence. For this booster compound, EF β-sheet amino acid sequence is localized "approximately " in the middle of the amino acid sequence of polypeptide (i), although the FG loop amino acid sequence, at the C-terminal end, has 12 amino acid long residues more than the one of the DE loop localized at the N-terminal end.

Generally speaking, a polypeptide (i) type booster compound according to the present invention is at least 30-amino acid long, and at most 195-amino acid long and most preferably at most 180-amino acid long.

So, a polypeptide (i) type booster compound according to the present invention is at least 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190 or 195-amino acid long.

According to a first preferred embodiment of an immune booster compound comprising a polypeptide (i) such as defined hereabove, the sequence of said polypeptide (i), having a length of "n " amino acids, is a

sequence of "n" consecutive amino acids of a corresponding sequence contained in the fiber protein knob domain of the considered adenovirus. Hence, there is an amino acid sequence identity between the booster compound of polypeptide (i) type sequence and the corresponding part sequence of the fiber protein knob domain of the considered adenovirus.

According to a second preferred embodiment, polypeptide (i), having a length of "n" amino acids, comprises a "x" consecutive amino acid sequence of the knob domain and the EF β-sheet sequence as well as one or two additional amino acid sequence(s), the additional amino acid sequence total length being "n–x" amino acids, localized at the N-terminal end and/or at the C-terminal end of the knob domain sequence containing "x " amino acids. It has to be understood that "n" is an integer ranging from 30 to 195 and "x" is an integer ranging from 30 to "n-x".

In a polypeptide (i) type booster compound, the additional sequence(s), other than the partial sequence of the knob domain of the fiber protein that comprises the EF β-sheet sequence, may be peptide sequences that can be detected with specific antibodies for such a peptide, that will therefore be used as a marker.

According to another aspect, additional sequences may be sequences enabling an easier purification of the booster compound after its synthesis, either by means of a chemical synthesis or a gene recombination synthesis. As an illustration of this aspect, additional sequences are selected from polyhistidine sequences, for example sequences comprising from 4 to 10, and preferably 6, histidine residues. These sequences may also comprise peptides or polypeptides intended to facilitate booster compound purification, such as GST.

Most preferably, the "x" amino acid additional sequence is localized on the C-terminal side of the fiber protein knob domain sequence of the considered adenovirus.

According to another embodiment, a polypeptide (i) type booster compound of the present invention comprises an amino acid sequence of an adenovirus fiber protein knob domain which comprises, from the N-terminal end towards the C-terminal end, the D β-sheet, the DE peptide loop, the EF β-sheet and the FG peptide loop of the fiber protein knob domain of the considered adenovirus. For the serotype 5 adenovirus knob

domain, such a polypeptide (i) has an amino acid sequence beginning with amino acid at position 454 and ending with amino acid at position 515 of the complete fiber protein, as illustrated in figure 6A.

According to a still further preferred embodiment, a polypeptide (i) type booster component according to the present invention comprises a knob domain amino acid sequence which comprises, from the N-terminal end towards the C-terminal end, respectively, the DE peptide loop, the EF β-sheet, the FG peptide loop and the G β-sheet.

As an illustration, for the adenovirus serotype 5 knob domain, such a polypeptide (i) comprises the sequence beginning with amino acid at position 463 and ending with amino acid at position 521 of the complete fiber protein, as illustrated in figure 6.

According to a still further preferred embodiment, a polypeptide (i) type booster compound comprises, from the N-terminal end towards the C-terminal end, respectively D β-sheet, DE peptide loop, EF β-sheet, FG peptide loop and G β-sheet.

As an illustration, for the adenovirus serotype 5 knob domain, such a polypeptide (i) comprises the amino acid sequence beginning with amino acid at position 454 and ending with amino acid at position 521 of the complete fiber protein.

According to a still further specific embodiment of a peptide booster compound of the invention, polypeptide (i) or analogous peptide (ii) also comprises, in addition to the fiber knob domain amino acid sequence, the last repeating sub-unit of the fiber shaft as well. Such a peptide booster compound according to the present invention comprising the fiber shaft last repeating sub-unit may have a high structural stability and block part of the knob domain in its native conformation and under the form of a peptide trimer.

As an illustration of this specific embodiment of a peptide booster compound of the invention which comprises an adenovirus serotype 5 fiber protein-derived polypeptide (i), said polypeptide (i) comprises the amino acid sequence beginning with amino acid residue at position 380 and ending with amino acid residue at position 581 of the amino acid complete fiber protein sequence.

According to a preferred characteristic of a booster compound according to the present invention, polypeptide (i) comprises part of the fiber protein knob domain of an adenovirus that is a human adenovirus.

Advantageously, the human adenovirus is selected from adenoviruses belonging to sub-group B, which comprises Ad3, Ad7, Ad11, Ad14, Ad16, Ad21, Ad34 and Ad35 adenoviruses, or from adenoviruses belonging to the sub-group C, which comprises Ad1, Ad2, Ad5 and Ad6 adenoviruses.

Preferably, the human adenovirus is selected from the group consisting in 12 18, 31, 3, 7, 11, 14, 16, 21, 34, 35, 1, 2, 5, 6, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33, 36, 37, 38, 39, 42, 43, 44, 45, 46, 47, 48, 49, 4, 40 and 41 serotype adenoviruses.

Preferably, a peptide booster according to the invention is derived from the fiber protein knob domain of an adenovirus selected from Ad5, Ad3 and Ad12.

Complete fiber proteins of Ad5, Ad3 and Ad12 adenoviruses correspond to the amino acid sequences SEQ ID N°1, SEQ ID N°2 and SEQ id N°3, respectively.

The knob domain of Ad5 adenovirus fiber protein begins with amino acid residue at position 400 of the SEQ ID N°1 sequence.

The knob domain of Ad3 adenovirus fiber protein begins with amino acid residue at position 132 of the SEQ ID N°2 sequence.

The knob domain of Ad12 adenovirus fiber protein begins with amino acid residue at position 409 of the SEQ ID N°3 sequence.

Ad5, Ad3 and Ad12 adenovirus fiber protein crystal structures have been described by Xia and al. (1994), Dumort and al. (2001) and Bewley and al. (1999), respectively.

According to a still further preferred aspect of a booster compound according to the present invention, this one is characterized in that polypeptide (i) comprises an amino acid sequence selected from following sequences:

- the sequence beginning with amino acid at position 463 and ending with amino acid at position 515 of SEQ ID N° 1 sequence;
- the sequence beginning with amino acid at position 195 and ending with amino acid at position 247 of SEQ ID N° 2 sequence;

- the sequence beginning with amino acid at position 472 and ending with amino acid at position 535 of SEQ ID N° 3 sequence.

As was already previously described, a specific embodiment of the booster compound according to the present invention, is a peptide (ii) analogous to polypeptide (i) such as defined hereabove, the amino acid sequence of which comprises, as compared to said polypeptide (i) sequence, at least one amino acid substitution or at least one amino acid deletion, said analogous peptide retaining the EF double β-sheet structure.

Most preferably, the amino acid substitution(s) or deletion(s), as compared to the polypeptide (i) sequence is or are localized in the analogous peptide (ii) part that is derived from the sequence of the adenovirus knob domain fiber protein contained in the parent polypeptide (i).

Preferably, the booster analogous peptide (ii) comprises 2,3,4,5,6,7,8,9 or 10 amino acid substitutions or deletions, as compared to the amino acid sequence of the parent polypeptide (i).

According to a still further embodiment of an immune booster compound according to the present invention, said booster compound is characterized in that polypeptide (i) or analogous peptide (ii) is a cyclic polypeptide.

The booster peptides (ii) comprising in their sequence one or more amino acid difference(s) as compared to the corresponding sequence comprised in the natural fiber protein knob domain of the considered adenovirus, have nevertheless booster properties, that is to say immature dendritic cell maturation-inducing properties, in a comparable extent as the parent polypeptide (i) they are derived from.

The present invention generally relates to booster peptides derived from the fiber protein knob domain of an adenovirus that present the same booster activity as booster peptides specifically described in the present specification.

As illustrated in the examples, the property of a booster compound according to the present invention consisting in inducing immature dendritic cell maturation may be easily controlled by the one skilled in the art, for example by determining the expression level of class I or class II

MHC molecules or the expression level of dendritic cell maturation-specific markers, such as CD40, CD80 and CD86 molecules.

The other tests described in the examples may also be used by the one skilled in the art to control the dendritic cell maturation booster ability of a compound according to the present invention, such as:

(i) the booster compound ability to stimulate immature dendritic cells to induce a mixed lymphocyte reaction in presence of allogenic CD4+ T-cells;

(ii) the ability of dendritic cells stimulated with a booster compound of the invention, and charged with an antigen, to induce an immune response by inducing CD8+ T-cells proliferation specific to said antigen, and even more specifically CD8+ T-cells secreting gamma interferon.

(iii) the ability of a booster compound according to the present invention to stimulate dendritic cell-induced IL-12 and TNFα production.

Preferably, in the sequence of an analogous peptide (ii), an amino acid comprised in the parent polypeptide (i) sequence is substituted with an amino acid belonging to the same amino acid class, selected from following classes: acidic amino acids (D, E), basic amino acids (K, R, H), non polar amino acids (A, V, L, I, P, M, F, W) or not charged polar amino acids (G, S, T, Y, N, Q).

Preferably, the analogous peptide (ii) part derived from the knob domain sequence of the parent polypeptide (i) being (x) amino acid long, has an amino acid identity with the (x) length corresponding sequence of the parent polypeptide (i) of at least 80%, advantageously of at least 95% and most preferably of at least 98%.

The "identity percentage" between two amino acid sequences, in the context of the present invention, is obtained by comparing the two optimally aligned sequences through a comparison window.

The amino acid sequence part in the comparison window may thus comprise additions or deletions (for example "gaps") as compared to the reference sequence (which does not comprise these additions or deletions) so as to obtain an optimal alignment between the two sequences.

The identity percentage is calculated by determining the number of positions where an identical amino acid residue is observed for the two

compared sequences, then by dividing the number of positions where there is an identity between the two amino acid residues by the total number of positions in the comparison window, then by multiplying the result with hundred so as to obtain the amino acid identity percentage between the two sequences.

The sequence optimal alignment for comparison may be effected by means of computer programs using known algorithms.

Most preferably, the sequence identity percentage is determined by means of CLUSTAL W software (1.82 version), parameters being defined as follows: (1) CPU MODE = ClustalW mp; (2) ALIGNMENT = "full "; (3) OUTPUT FORMAT = "aln w/numbers "; (4) OUTPUT ORDER = "aligned "; (5) COLOR ALIGNMENT = "no "; (6) KTUP (word size) = "default "; (7) WINDOW LENGTH = "default "; (8) SCORE TYPE = "percent "; (9) TOPDIAG = "default "; (10) PAIRGAP = "default "; (11) PHYLOGENETIC TREE/TREE TYPE = "none "; (12) MATRIX = "default "; (13) GAP OPEN = "default "; (14) END GAPS = "default "; (15) GAP EXTENSION = "default "; (16) GAP DISTANCES = "default "; (17) TREE TYPE = "cladogram " and (18) TREE GRAP DISTANCES = " hide ".

A peptide booster compound according to the present invention may be synthesized by ordinary synthetic chemistry methods, either homogenous chemical syntheses in solution or in solid phase.

As an illustration, the one skilled in the art can use solution polypeptide synthesis methods described by HOUBEN WEYL (1974).

A peptide booster compound according to the present invention may also be chemically synthesized in a liquid or solid phase by successively coupling the different amino acid residues (from the N-terminal end towards the C-terminal end in liquid phase, or from the C-terminal end toward the N-terminal end in solid phase). The one skilled in the art can especially use peptide synthesis method in solid phase described by Merrifield (1965a; 1965b).

According to another aspect, a peptide booster compound according to the present invention may be synthesized by genetic recombination, for example according to a production method comprising following steps consisting in:

(a) preparing an expression vector wherein a nucleic acid encoding the peptide booster compound of the invention was inserted, said vector also comprising regulating sequences needed for expressing said nucleic acid into a selected host cell;

(b) transfecting a host cell with the recombinant vector obtained in step (a);

(c) culturing in a suitable culture medium the host cell transfected in step b);

(d) recovering the culture supernatant of the transfected cells or the cell lysate of said cells, for example by sonication or by osmotic shock; and

(e) separating or purifying, from said culture medium, or from cell lysate pellet, the recombinant peptide booster compound of the invention.

To produce a recombinant peptide booster compound of the invention, the one skilled in the art may especially refer to methods for preparing recombinant vectors, cell transfection and purification methods that are described in the examples.

Most preferably, a baculovirus type vector is used for infecting Sf9 cells, such as described in the examples.

In order to purify an immune booster peptide compound according to the present invention which was prepared from host cells transfected or infected by a recombinant vector encoding said peptide booster compound, the one skilled in the art can advantageously carry out purification methods described by Molinier-Frenkel (2002), by Karayan and al. (1994) or by Novelli and al. (1991).

As already previously mentioned in the present description, since mature dendritic cells do contribute to improve the cell-mediated immune responses, any peptide booster compound according to the present invention may be used in combination with an antigen against which an immune response is expected.

A peptide booster compound according to the present invention may be associated, in order to induce an immune response, in humans or animals, indifferently with a peptide antigen or with an antigen of the carbohydrate type, for example a carbohydrate type antigen identical or similar, as regards its antigenic recognition, to an antigen that is specifically expressed by tumoral cells.

By definition, the peptide booster compound according to the present invention may be associated with any type of antigen against which an immune response is needed.

According to a specific embodiment, a peptide booster compound of the invention is chemically coupled to one or more antigen(s) against which an immune response is expected, in the form of a peptide booster/ peptide antigen conjugate or in the form of a peptide booster-antigen carbohydrate conjugate.

So, it is also an object of the present invention to provide an immunoconjugate comprising a peptide booster compound according to the present invention, which is covalently bound to an antigen against which an immune response is needed.

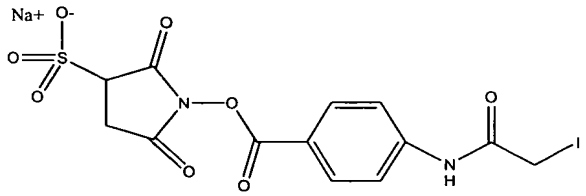
It is a further object of the invention to provide an immune booster composition comprising a peptide booster compound such as defined hereabove in the present description, in combination with at least one physiologically compatible excipient.

In a specific embodiment of the immunoconjugate according to the invention, the booster compound and the antigen are directly covalently bound to each other, for example via a peptide -CO-NH- bond.

However, in order to introduce into the immunoconjugate structure some flexibility, and especially to allow some reciprocal spatial mobility within the immunoconjugate for both the booster compound and the antigen, a peptide conjugate is preferred, wherein the booster compound and the antigen are separated from each other, within said conjugate, by a spacer chain.

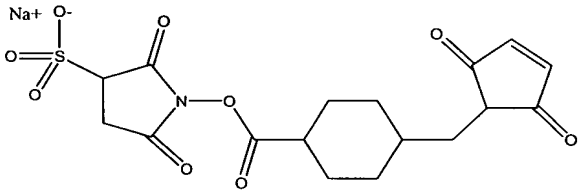
According to an immunoconjugate first preferred embodiment, the booster compound and the antigen are separated from each other, within said conjugate, by a spacer chain selected from SMCC or SIAB, both being bifunctional compounds.

SIAB compound, described by Hermanson G.T. (1996, Bioconjugate techniques, San Diego: Academic Press, pp 239-242), is the compound having following formula (I):

(I) 

SIAB compound comprises two reactive groups, respectively a iodoacetate group and a sulfo-NHS ester group, these groups respectively reacting with amino and sulfhydryl groups.

SMCC compound, described by Samoszuk M.K. and al. (1989, Antibody, Immunoconjugates Radiopharm., 2(1): 37-46), is the compound having following formula (II):

(II) 

SMCC compound comprises two reactive groups, respectively a sulfo-NHS ester group and a maleimide group, reacting respectively with an amino group and a sulfhydryl group.

According to a second preferred embodiment, the immuno-conjugate comprises a spacer chain comprising a linear spacer peptide. A 3 to 30 amino acid-long linear spacer peptide will be preferably selected, advantageously having a length of from 5 to 20 amino acids and most preferably from 7 to 15 amino acids.

Preferably, the linear spacer peptide substantially, or even exclusively, comprises positively or negatively charged amino acids at pH 7.0 in order to increase the overall hydrophilicity of said peptide immunoconjugate. It will be appreciated that one should avoid using spacer peptides comprising hydrophobic amino acids. Preferably, the spacer peptide is characterized in that it comprises a poly-(lysine) chain, the length of which ranges from 3 to 30 lysine residues, advantageously from 5 to 20 and most preferably from 7 to 15 lysine residues.

According to a still further immunoconjugate embodiment according to the present invention, the booster compound and the antigen

are separated from each other, within said peptide conjugate, by a spacer chain comprising a branched spacer peptide, preferably a poly(lysine) oligodendrimeric structure, such as described for example by Basak and al. (1995).

In this last immunoconjugate embodiment according to the present invention, said peptide conjugate may respectively comprise several copies of the booster compound and/or of the antigen per conjugate molecule, advantageously from 2 to 8 copies of the booster compound and/or of the antigen, preferably at most 4 copies of the booster compound and/or of the antigen, per conjugate molecule.

It is another object of the present invention to provide a peptide booster compound such as previously defined in the description, to be used as a booster active agent in an immunogenic composition or in a vaccinal composition.

As used herein, an "immunogenic composition" means a composition comprising a peptide booster compound such as defined hereabove in combination with at least one antigen against which a cell-mediated immune response is needed, in order to produce specific antibodies against said antigen.

As used herein, "vaccinal composition" means a composition comprising a peptide booster compound such as defined hereabove, in combination with at least one antigen against which a cell-mediated immune response is needed, in order to prevent or to treat a disease, more particularly a disease induced by a pathogenic agent of the viral, fungal or bacterial type, or even a tumor.

The present invention also relates to the use of a peptide booster compound such as defined hereabove for preparing an immunogenic or a vaccinal composition.

It also relates to an immunogenic composition or a vaccinal composition comprising a peptide booster compound such as defined hereabove, in combination with at least one antigen.

In an immunogenic composition or in a vaccinal composition of the invention, the antigen may be used as a mixture with the peptide booster compound of the invention.

According to a further embodiment, the peptide booster compound and the antigen comprised in a vaccinal composition or in an immunogenic composition of the invention come as an immunoconjugate such as defined hereabove.

It has been demonstrated in the examples that dendritic cells stimulated with a peptide booster compound according to the present invention and charged with a determined antigen, here LCMV GP33 peptide, were able to induce, in animal, a cytotoxic immune response by stimulating CD8+ T-cells specific to the GP33 antigen.

It has been shown more particularly that mature dendritic cells stimulated by a peptide booster compound according to the present invention and presenting to the immune system cells the interesting antigen were able to induce an immune response of the cytotoxic type causing the splenocyte rejection presenting on their surface the interesting antigen.

In an immunogenic composition or in a vaccinal composition according to the present invention, any type of antigen may be used since, in any case, and whatever the antigen(s) is or are, the suitable peptide of the invention will exert its dendritic cell-activating activity.

Preferably, an immunogenic composition or a vaccinal composition according to the present invention comprises said peptide booster compound such as defined hereabove in an amount ranging from 10 nanograms to 1 milligram, preferably from 100 nanograms to 100 micrograms, and most preferably from 100 nanograms to 10 micrograms.

There are amongst the antigens that may be comprised in an immunogenic composition or in a vaccinal composition according to the present invention, in combination with a peptide booster compound, following bacterial antigens derived especially from *B. pertussis, Leptospira pomona, S. paratyphi AB, C. diphtheriae, C. tetani, C. botulinum, C. perfringens, C. feseri, B. anthracis, P. pestis, P. multocida, V. cholerea, Neisseria meningitidis, N. gonorrheae, Hemophilus influenzae, Treponema pollidum.*

Antigens also may be viral antigens such as antigens derived from virus poliovirus, adenovirus, para influenza virus, respiratory syncytial

virus, influenza virus, encephalomyelitis virus, Newcastle disease virus, pox virus, rabies virus.

Antigens may also be antigens derived from any allergen, such as flower pollen extract or herb extract-derived allergens, purified allergens from house dust, etc.

Amongst interesting viral antigens, there are also antigens derived from papillomavirus proteins, especially human papillomavirus proteins, and more particularly from L1, E6 and E7 proteins, especially from HPV-16 strains.

Other illustrative viral antigens include antigens derived from the human immunodeficiency virus (HIV) proteins, more particularly from HIV-1, and most preferably derived from HIV-1 virus ENV protein.

Other interesting antigens that may be comprised in an immunogenic composition or in a vaccinal composition according to the present invention are tumoral antigens, that is to say antigens expressed by cancerous cells, these antigens being either peptidic or carbohydrate in nature.

As understood, immunogenic compositions or vaccinal compositions according to the present invention are especially to be used in the treatment both curative and prophylactic, of cancers, especially virus-induced cancers as for example ATL (acute T leukemia) induced by HTLV1 virus, or papillomavirus-induced cancer of cervix uteri, or Burkitt's lymphom or Kaposi's sarcoma induced by herpes family viruses, respectively Epstein-barr (EBV) and HHV8, as well as in the treatment of AIDS or to prevent or to treat allergic inflammatory reactions.

According to a still further aspect, it is another object of the invention to provide a method for immunizing a human or an animal, more specifically a mammal, against an interesting antigen, said method comprising a step during which a human or an animal is given an immunogenic composition or a vaccinal composition such as defined hereabove.

An immunogenic composition or a vaccinal composition according to the present invention is administrated to a human or an animal, for example to patients, said composition coming in a form adapted to the systemic or mucosal administration, for example via the intranasal route, in

a therapeutically effective amount, to a subject in need of such a treatment. An immunogenic composition or a vaccinal composition according to the present invention is in solid or liquid form, more particularly in the form of an oil-in-water emulsion wherein the interesting antigen(s) is or are dispersed. To formulate an immunogenic composition or a vaccinal composition according to the present invention in the form of an oil-in-water emulsion, the one skilled in the art can use a SPT type emulsion such as described in the book "Vaccin Design, The subunit and adjuvant approach", page 147, [M. POWELL, M. Newman Ed., Plenum Press, (1995)] as well as the MF59 emulsion described on page 183 of the same reference.

As used herein "a physiologically compatible excipient" means a liquid or solid filler, a diluent or any other physiologically non active substance and presenting a high safety for the patient which may be used for systemically or topically administrating, for example on mucosae, an immunogenic composition or a vaccinal composition according to the present invention.

Such physiologically compatible excipients are described in detail especially in the 4th edition "2002" of the European Pharmacopeia, as well as in USP 26-NF21 edition published in November 2002.

According to a still further aspect, the invention also relates to a method for the *in vitro* maturation of human or animal immature dendritic cells, in which immature dendritic cells are stimulated with a peptide booster compound such as defined in the present description.

It is hence a further object of the invention to provide a method for *in vitro* maturating human or animal immature dendritic cells, characterized in that it comprises following steps consisting in:

(a) *in vitro* culturing in a suitable culture medium a cell population enriched with human or animal immature dendritic cells;

(b) incubating the cells cultured in step (a) with a peptide booster component or with a booster composition such as defined in the present description, for a time sufficient to induce dendritic cell maturation.

The cell population enriched with human or animal immature dendritic cells used in method step a) may be obtained from a bone marrow sample or from a human or animal blood sample, according to methods

well known by the one skilled in the art, as for example the method described by Mayordomo and al. (1995).

To purify and cultivate dendritic cells, the one skilled in the art may also refer to the method described by Steinam and Young (1991). The one skilled in the art may also refer to many bibliographic references about dendritic cell purification and culture described in the PCT application published under the number WO 98/23728, and more specifically on pages 1 to 3.

To obtain a cell population enriched with human or animal immature dendritic cells, the one skilled in the art may also refer to the method described in the examples.

In step b) of above maturation method, immature dendritic cells are incubated with a peptide booster compound final concentration ranging from 10 nanograms per ml to 1 μg/ml, preferably from 50 nanograms per ml to 1 μg/ml.

In step b) of above maturation method, immature dendritic cells are incubated for a time ranging from 1 hour to 48 hours, with the selected peptide booster compound final concentration.

The invention also relates to a cell population enriched with mature dendritic cells charged with a booster compound or with a booster composition such as defined hereabove. A booster compound according to the present invention may so be detected, for example using an antibody specifically directed against this booster compound, indifferently in the cytoplasm or in the membrane surface of those dendritic cells that may be obtained by means of the maturation method such as defined hereabove.

Mature dendritic cells obtained by means of the hereabove maturation method are characterized in that they simultaneously express MHC class I and class II molecules as well as mature dendritic cell-specific CD40 CD80 and CD86 markers.

It is a further object of the present invention to provide an immune booster cell composition, characterized in that it comprises a mature dendritic cell population charged (i) with a booster compound or with a booster composition such as defined hereabove, and (ii) charged with the interesting antigen that may be obtained by means of the hereabove maturation method.

Preferably, a dose of the immune booster cell composition such as defined hereabove to be administrated to patients, comprises a mature dendritic cell number ranging from 10^6 to 10^9 syngenic mature dendritic cells, advantageously from 10^7 to 10^9 syngenic cells.

In a booster cell composition according to the present invention, mature dendritic cells are preferably suspended in a saline liquid medium needed for them to survive for several hours, preferably at least three hours.

Most preferably, mature dendritic cells are suspended in a suitable culture medium comprising all the nutrients needed for their long term survival, for example for several days, preferably for at least 2 days.

The invention also relates to a method for preparing an immunogenic cell composition, characterized in that it comprises following steps consisting in:

a) *in vitro* culturing in a suitable culture medium a cell population enriched with human or animal immature dendritic cells;

b) incubating the cells cultured in step a) with a peptide booster compound or with a booster composition such as previously defined, for a time sufficient to induce dendritic cell maturation;

c) adding to cells cultured in step b) at least one interesting antigen against which an immune response is needed.

According to the above method, the dendritic cell incubation step c) with at least one interesting antigen may indifferently be simultaneous to step b) wherein the peptide booster compound is incubated with the cells, or on the contrary be subsequent to step b). Nevertheless, and most preferably, steps b) and c) are effected simultaneously.

It is also an object the invention to provide a method for preparing an immunogenic cell composition characterized in that it comprises following steps consisting in:

a) *in vitro* culturing in a suitable culture medium a cell population enriched with human or animal dendritic cells;

b) incubating the cells cultured in step a) with an immunoconjugate such as previously defined, for a time sufficient to induce dendritic cell maturation.

In the above methods for preparing an immunogenic cell composition, the final concentration for the addition to dendritic cells may

vary depending on the nature and on the molecular weight of the interesting antigen. For example, LCMV GP33 peptide is added to the dendritic cells. For each interesting antigen, the one skilled in the art should be able to adapt the final concentration which has to be added in step c) (first method), or the final concentration of immunoconjugates which has to be added in step b) of the method (second method), thanks to his general technical knowledge concerning the charging of dendritic cells with an interesting antigen.

General conditions of the above immunogenic cell composition preparation methods are otherwise identical to those used for the dendritic cell maturation method which has been previously described.

The one skilled in the art may also refer to the examples of the present patent specification, wherein all details are given to prepare an immunogenic cell composition according to the present invention.

It is another object of the invention to provide an immunogenic cell composition, characterized in that it comprises a mature dendritic cell population charged with the interesting antigen obtained using the methods for preparing the same as described hereabove.

Mature dendritic cells charged with the interesting antigen are characterized in that they express MHC class I and class II molecules, as well as CD40, CD80 and CD86 mature dendritic cell-specific markers. Moreover, these cells present on their surface peptide fragments of the interesting antigen against which an immune response is needed

The invention also relates to a method for immunizing a human or an animal body system against an interesting antigen, said method comprising a step during which a booster cell composition such as defined hereabove is given to a patient, before, during or after the interesting antigen administration step.

It is also an object of the present invention to provide a method for immunizing a human or an animal body system against an interesting antigen, said method comprising a step during which an immunogenic cell composition according to the present invention is given to the patient.

The present invention will be further illustrated in the following examples:

EXAMPLES:

A. MATERIAL and METHODS

A.1 Adenovirus (Ad) and adenovirus (Ad) capsid protein preparation, including the peptide booster compounds according to the present invention.

Ad5E1 adenovirus is a serotype 5 adenovirus replication-defective and deleted in both the early regions E1 and E3. Ad51E1 adenovirus carries wild type (WT) fiber proteins (Molinier-Frenkel and al., 2002).

Ad5E1°Δ knob adenovirus, deleting mutant for fiber protein, is derived from Ad5E1 adenovirus by inserting a "opal" stop codon in the gene encoding the fiber, downstream the extrinsic trimerization signal contained in the shaft of the fiber (Magnusson and al., 2001; Hong and al., 2003).

The resulting fiber does not possess the whole knob domain ("knobless") and only possesses the tail domain as well as the seven repeating units of the shaft N-terminal end, that is to say the fiber protein amino acid residues 1 to 157.

Figure 1 shows the fiber schematic structure of Ad5E1 and Ad5E1°Δ knob adenoviruses. Ad5E1° and Ad5E1°Δ knob virions were isolated by means of isopycnal ultracentrifugation in cesium chloride continuous gradient (Molinier-Frankel and al., 2002). The Hexon capsid proteins (hereafter abbreviated "Hx") and penton capsomeres (hereafter abbreviated "Pn", for the combination penton base + fiber) were isolated from HeLa cells infected with Ad5 WT wild serotype 5 adenovirus.

Wild serotype 5 adenovirus penton-base proteins (hereafter also abbreviated "Pb"), fiber proteins (hereafter also abbreviated "Fi") and fiber knob domain proteins were isolated as recombinant proteins from baculovirus-infected Sf9 cells.

Four Ad5 adenovirus mutated fiber proteins were also analyzed, carrying deletions with varying lengths in the knob domain (Santis and al., 1999), respectively mutant proteins named Fi Δ402-480, FiΔHI, FiΔEF and FiΔLT485 – 486 in figure 6B. These mutated fiber proteins were produced as recombinant proteins in the Sf9 cells.

Adenovirus proteins were purified according to a procedure described by Molinier-Frenkel and al. (2002), Karayan and al. (1994) and

Novelli and al. (1991). Briefly, adenovirus proteins were purified using a method comprising the three following steps:

(i) precipitation with ammonium sulfate,

(ii) anion exchange high-performance liquid chromatography (HPLC) and

(iii) concentration-ultrafiltration step using concentration membranes with a cut-off threshold of 100 kDa.

The protein samples were analyzed using conventional SDS 12%-polyacrylamide gel electrophoresis methods (Molinier-Frenkel and al., 2002) and immuno-empreinte (Karayan and al., 1994).

Potential endotoxins existence in protein and adenovirus preparations were searched for, by means of the *Limulus polyphemus* amoebocyte lysate test (E-TOXATE®; sigma), using the lipopolysaccharide (LPS) of E. coli of serotype 055:B5 as standard control.

Endotoxin concentration in the different protein batches never exceeded 30 pg/ml at doses used to stimulate dendritic cells. With such concentration, Hx protein samples never induced dendritic cell maturation at a level exceeding the non stimulated dendritic cell background (see figure 2). Adenovirus virions purified on CsCl gradient were totally free from any endotoxin-detectable contamination.

A.2- Dendritic cell preparation and culture.

The method has been adapted from the technique as described by Mayordomo and al. (1995). C57BL/6 ($H2^b$) mice marrow bone cells, (Harlan, Gannat, France) deleted in lymphocytes were cultured overnight in a complete culture medium (RPMI 1640 additioned with 10 % fetal calf serum (FCS), 2 mM L-glutamine, 50 µM-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin). Non adhering cells were collected and suspended in the complete culture medium again in presence of 2000 U/ml recombinant GM-CSF (rGM-CSF, R & D System, Mineapolis, Minnesota) and 100 U/ml recombinant IL4 (rIL4, R & D System). The culture medium was replaced at day 4. At day 6, non adhering cell aliquots were suspended again at $3x10^6$/ml density in a PBS buffer with 1% FCS. Cells were then incubated for an hour at 37°C (i) without (non stimulated dendritic cells; NS-DC), or (ii) with Ad5 capsid components, or (iii) with corresponding chromatographic fractions obtained from the Sf9 cells extracts infected with

an empty baculovirus vector (dendritic cells stimulated by the empty vector; MS-DC), as indicated in the figure legends.

After one hour incubation, the cell concentration was adjusted at 3×10^5 cells per ml with GM-CSF added-complete medium. Control cells were incubated with 1 µg/ml LPS from *E.coli* (sigma).

At day 8, cells were recovered and analyzed using flow cytometry, mixed lymphocyte reactions (MLRs) and immunization techniques. At day 8, culture supernatants were collected. For the experiments described and illustrated in figure 4A, dendritic cells were purified using magnetic beads conjugated with a mouse anti-CD11c monoclonal antibody (Miltenyi Biotech).

Dendritic cells were washed in a PBS buffer with 1 % FCS. After incubation with an anti-FCII/IIIR antibody (2.4G2; Pharmingen), cells were incubated with the following various monoclonal antibody combinations (all marketed by Pharmingen): anti-I-A^b (AF6-120.1) and anti-CD40(3/23), conjugated with PE anti-CD11c (HL3) and anti-CD80(16-10A1) conjugated with FITC, anti-H2-D^b(28-8-6) and anti-CD86(GL1) biotinylated + streptavidin-PerCP (Becton Dickinson). Mouse anti-fiber-tail monoclonal antibodies were also used (4D2.5; HONG and al., 1997). For staining the CAR receptor, dendritic cells at day 6, CHO lineage cells (ATCC N°CCL-61) and Hela lineage cells (CCL-2 ATC CCl2) and CHO were labeled with a monoclonal antibody from anti-CAR E1.1 ascite, as well as with a control ascite. A counter-staining was effected using biotinylated rate anti-mouse IgG and a streptavidin-PE conjugate. Flow cytometry analyses were effected on a FACSCalibur equipment (Becton Dickinson).

A.3 *in vitro* functional tests

For mixed lymphocyte reactions (MLRs), dendritic cells at day 8 were distributed at increasing doses in culture wells and co-cultured 4 days long with allogenic (Balb/c, $H2^d$) CD4+ purified splenocyte aliquots (2×10^5 cells/well). CD4+ T-cell proliferation was measured by incorporating ^{3}H-thymidine (1µCi/well) for the co-culture step last 18 hours. To measure IL12 and TNFα production, dendritic cell culture supernatants at day 8 were tested using ELISA IL12p70 and TNFα kits (Pharmingen).

A.4 Peptides

33-41 peptide of LCMV glycoprotein (KAVYNFATM) and 366-374 peptide of influenza virus nucleoprotein (ASNENMETM), hereafter abbreviated GP33 and NP366, respectively, are marketed by NEOSYSTEM (Strasbourg, France).

A.5 Immunization assays and rejection tests for CFSE-labeled splenocytes.

C57BL/6 mice were subcutaneously (s.c.) immunized in the flank with $2.5x10^5$ dendritic cells maturated in presence of various adenovirus capsid components, then charged with GP33 or NP366 (10^{-6}M). Ten days later, mice were given intravenous injection of $3x10^7$ syngenic splenocytes labeled with fluorescent 5-6-carboxyfluorescein diacetate succinimidyl ester dye (CFSE; Molecular Probes) as described by OEHEN and al. (1997). $CFSE^+$ donor cell percentage within splenocytes or receptive peripheral blood lymphocytes (PBLs) was determined using a flow cytometry analysis (FACS). Donor cell rejection was calculated using following formula:

$CFSE^+$ cells % in immunized mice/ % $CFSE^+$ cells in naive mice] ratio x 100.

A.6 ELISPOT-IFNγ Assays.

Nitrocellulose microtiter plates (Millipore) were coated with a rat anti-mouse IFNγ antibody (R4-6A2; Pharmingen), then the wells were washed and saturated with complete medium. Freshly isolated splenocyte aliquots (10^6 cells/well), or freshly isolated peripheral blood lymphocyte (PBLs) aliquots (2×10^5 cells/well), in triplicates, were added to the culture wells in complete medium containing 30 U/ml human recombinant IL2 (hrIL2, Boehringer) and 10^{-6} M GP33 or NP366 peptides. After 20 hours culture at 37°C, IFN-γ secreting cells form spots (SFC). Cells were counted according to the technique described by Molinier-Frenkel and al. (2002). Values obtained with NP366 peptide were subtracted from the triplicate test mean value obtained with GP33 peptide.

A.7 Cytotoxicity tests

Mice splenocytes that have been immunized with dendritic cells were cultured for 4 days with 10^{-6} M GP33 peptide. These cells were then tested in duplicate with various amounts of effector cells for a fixed amount

of ^{51}Cr–labeled (10^{-4} µCi/cell) EL-4 target cells which were incubated with GP33 or NP366 peptide. After five hours culture at 37°C, culture supernatants were collected and radioactivity was measured by means of a Top-Count type device (Packard Instruments). In control samples, EL4 target cells were incubated with the medium alone so as to determine ^{51}Cr spontaneous release level and with 2% alkyl trimethylammonium bromide (Sigma) so as to determine ^{51}Cr total release level.

B. RESULTS

EXAMPLE 1: Effect of capsid components isolated from adenovirus serotype 5 (Ad5) on dendritic cell phenotype.

Immature dendritic cells were incubated with penton (Pn) or hexon (Hx) capsomeres which were purified from Ad5-infected HeLa cells. Most of dendritic cells stimulated with Hx had a mature phenotype low level, as compared to the non stimulated dendritic cells (NS-DC), with a low expression of MHC class II molecules and CD40, CD80 (B7.1) and CD86 (B.7.2) molecules, as illustrated in figure 2A. On the contrary, dendritic cells stimulated with penton (Pn) substantially expressed a mature phenotype, similar to the phenotype observed for LPS-stimulated dendritic cells, with a high expression level for the various markers, as illustrated in figure 2A.

Since adenovirus Pn capsomere comprises two structural entities, i.e. the penton base (Pb) and the fiber (Fi), respectively, following experiments were developed so as to determine which amongst these two constitutive proteins, either Pb or Fi, respectively, was responsible for dendritic cell maturation.

Dendritic cells were then stimulated with Pb or Fi recombinant proteins isolated from the extracts of insect infected Sf9 cells. Pn and Hx complete recombinant proteins were used as control samples.

Results obtained demonstrate that Fi protein is sufficient to reproduce the entire stimulating effect provided by Pn capsomeres, whereas Pb protein alone does not induce any detectable effect on dendritic cell maturation, as illustrated in figure 2B.

EXAMPLE 2: Role of the knob domain of the fiber in dendritic cell maturation by adenovirus and fiber protein.

As mentioned hereabove, the adenovirus fiber comprises three structural domains, respectively, from its N-terminal end towards its C-terminal end, the tail, the shaft and the knob. The knob domain was expressed as a recombinant protein, according to techniques described by NOVELLI and al. (1991) and by Hong and al. (1997). Dendritic cells which were incubated with the fiber knob domain expressed a mature phenotype, similar to that observed with dendritic cells stimulated with complete Fi fiber protein, as illustrated in figure 3A.

Dendritic cells stimulated with fiber knob domain are able to strongly induce allogenic CD4+ T-cells proliferation in a mixed lymphocyte reaction (MLR) assay as illustrated in figure 3B. Moreover, dendritic cell maturation level may vary depending on protein increasing concentrations of the fiber knob domain (figure 3B).

At the knob domain protein highest dose used (0.5 µg), dendritic cells stimulated by the knob domain stimulated allogenic $CD4^4$ T-cells more efficiently than non stimulated (NS) dendritic cell controls or than Hx protein-stimulated dendritic cells.

However, no detectable effect was obtained with the 0.02 µg knob protein dose. Interestingly, the mixed lymphocyte reaction (MLR) value for the 0.2 µg knob protein dose was similar to the value obtained with a 1 µg fiber protein dose. This result is compatible with the fact that the knob protein amount in a 0.2 µg knob protein sample approximately corresponds to the knob protein amount contained in a 1 µg fiber protein sample. The knob protein also induced a IL-12 and TNFα dose-related secretion, as illustrated in figure 3C.

It was tried then to know whether the knob domain was able to directly target dendritic cells and to induce the maturation of the same, without intermediate cell participation. To that end, CD11c+ cells were purified using magnetic beads coated with anti-CD11c antibodies, the purified CD11c+ cells having been used for maturation assays. Although non stimulated dendritic cells are able to increase the MHC molecule and costimulating molecule expression, knob domain-stimulated dendritic cells

express a phenotype significantly more mature than control cells do (figure 3A).

Moreover, it has been demonstrated that Fi recombinant protein was able to bind to immature dendritic cells, with 63% positive cells at culture day 6 (figure 3B). In this experiment, Cell-bound Fi protein was detected using an anti-tail monoclonal antibody, thus implying that the epitope contained in the tail could be accessed to, what suggests that the Fi protein binding to the cell surface occurred via the knob domain. These results highly suggest that dendritic cells represent direct targets for the knob domain of the adenovirus fiber protein.

To determine whether the Ad5 virion effect on the dendritic cell activation directly occurred through the fiber knob domain as well, comparative experiments were carried out using (ii) Ad5E1 vector, an Ad5 vector bearing wild (WT) Fi fiber projections or (ii) Ad5E1°Δ knob vector, an Ad5 vector without knob domain. During the incubation with Ad5E1 vector, an expression increase was observed in dendritic cell maturation markers (figure 3C), as was expected in view of Hirshowitz and al. (2000) and de Morelli and al. (2000) previous observations.

By using Ad5E1°Δ knob vector particle identical doses, it has been demonstrated that dendritic cells expressed a significantly less mature phenotype than they did after incubation with the Ad5E1 vector.

These results confirm that the fiber knob domain is the stimulating factor that is common to all viral active components, and that the knob domain carries the major determinants responsible for murine dendritic cell stimulation observed with Ad5 virions, the Pn complete capsomer, the complete fiber protein, and the recombinant protein of the knob domain of the isolated fiber.

EXAMPLE 3: Absence of binding of adenovirus fiber protein knob domain binding to the CAR receptor.

It has been extensively illustrated in the literature that dendritic cells do not express the CAR receptor.

However, the expression of this crucial protein for binding the knob domain to differentiated dendritic cells was analyzed by means of a flow cytometry (FACS) in the culture conditions used in the present study.

HeLa cells were used as positive control, that are known to express from about 10.000 to 30.000 CAR molecules/cell, and CHO lineage cells as negative control.

No CAR expression could be detected above the control ascite background on the immature dendritic cells collected before stimulation, in spite of a HeLa cell-highly specific staining, as illustrated in figure 5A.

Tests were performed so as to determine whether dendritic cell stimulation mechanism via the knob domain involved an effective adenovirus penetration as well as an expression of this gene. Serotype 5 adenoviral vector encoding a "Enhanced Green Fluorescent Protein" (EGFP) was used with two different infection index values (MOI) 10.000 and 30.000. As illustrated in figure 4C, a MOI index corresponding to 10.000 viral particles per cell induces indeed an efficient dendritic cell maturation. However, a significant expression of the EGFP fluorescent protein by the dendritic cells was only observed with the 30.000 particles per cell dose (figure 4C) which reveals a low "permissivity level" to infection by dendritic cell adenovirus.

EXAMPLE 4- Mapping of the fiber protein knob domain region responsible for dendritic cell maturation.

The induction of dendritic cell maturation was tested with four Ad5 adenovirus fiber protein mutants carrying deletions in the knob domain (figures 6A and 6B). The deletion present in the Δ402-481 recombinant fiber protein covers AB peptide loop, B and C β-sheets, CD peptide loop as well as the N-terminal end of the DG peptide loop. FiΔHI mutated recombinant fiber protein is only free from HI peptide loop. Two others recombinant mutated Fi proteins, FiΔEF and FiΔLT485-486, respectively, carry deletions in the short region of the EF anti-parallel double β-sheet (FiΔEF), or a deletion of both amino acid residues I485 and T486 which represent βF-sheet (FiΔLT485-486) (see Kirky and al., 2000 and Xia and al., 1993).

FiΔEF and FiΔLT 485-486 mutated Fi proteins come as trimeric fibers, whereas FiΔ402-481 and ΔHi mutated Fi proteins are defective for the fiber trimerization (see Santis and al., 1999).

Dendritic cell maturation activity of the wild (WT) knob domain was compared to mutant fiber proteins described in figure 6B, in a mixed lymphocyte reaction (MLR) test.

Four time less wild (WT) knob domain than mutated fiber protein was used to stimulate dendritic cells, in order to have in all assays approximately the same amount of knob domain.

As illustrated in figure 6C, FiΔHi mutant retained the whole dendritic cell maturation activity carried by the wild knob domain, thus implying that distal Hi peptide loop as well as the knob domain trimeric structure are not needed for inducing dendritic cell maturation.

The three other mutated Fi fiber proteins, FiΔ402-481, FiΔEF and FiΔLT 485-486, respectively, have not induce a dendritic cell maturation, thus suggesting that EF anti-parallel double β-sheet region, and more specifically F short β-sheet, is compulsary for the dendritic cell maturation effect.

To evaluate the *in vivo* effect, fiber protein knob domain-stimulated dendritic cells were compared to dendritic cells stimulated with other adenovirus components, as to their efficiency in inducing a $CD8^+$ T cell response specific to the GP33 peptide derived from LCMV glycoprotein, which is limited to D^b haplotype.

Vital fluorescent CFSE dye was used to easily follow-up the presence of the adoptively transferred splenocytes by means of a flow cytometry analysis and by controlling their destruction through specific $CD8^+$ T-cells induced by immunization via dendritic cells.

As illustrated in figure 7A, the CFSE-labeled spleen cell population charged with GP33 peptide decreased within 24 hours in blood of all immunized mice with dendritic cells stimulated by Pb, Hx proteins, or by the knob domain fiber protein and contacted with GP33 peptide.

However, a significantly more rapid cell rejection was observed in mice treated with fiber knob domain-stimulated dendritic cells, with a rejection rate similar to that observed in mice immunized with GP33 peptide emulsified in Freund's incomplete adjuvant (IFA). The cell rejection curve for mice immunized with fiber knob domain-stimulated dendritic cells and charged with the NP366 peptide is the same as the curve observed

in mice immunized with dendritic cells charged with GP33 peptide and brought in presence of Pb or Hx protein, or non-stimulated.

As illustrated in figure 7A, dendritic cells maturated with fiber knob domain are ten times as efficient in inducing syngenic splenocyte rejection than the dendritic cells stimulated by the other adenovirus components, as is suggested when comparing the effects obtained with immunizing with $3x10^4$ knob domain-stimulated dendritic cells, to the effects obtained after immunization with 2.5×10^5 Hx and Pb-stimulated dendritic cells.

As illustrated in figure 7A, CFSE+ cell analysis in the spleen of recipient mice, 10 days after the adoptive transfer, gave even more significant results: 5% CFSE+ cells remained in mice immunized with fiber knob domain-stimulated dendritic cells, whereas CFSE+ cells represent respectively 72% and 60% of the initial CFSE+ cells in mice immunized with dendritic cells stimulated by Pb and Hx proteins, respectively.

Ten days after the adoptive transfer of the CFSE-labeled splenocytes (which corresponds at day 20 following dendritic cell-induced immunization), the IFNγ-secreting $CD8^+$ T-cells number was evaluated by carrying out *ex vivo* ELISPOT assays.

As illustrated in figure 7B, there was neither detection of significant induction of GP33 peptide specific T-cells in mice immunized with Hx or Pb protein stimulated-dendritic cells, then charged with GP33 peptide, nor in mice immunized with fiber knob domain stimulated-dendritic cells and charged with the NP366 peptide.

On the other hand, a IFNγ-secreting T-cell significant amount was detected both in the spleen and in the blood of mice immunized with dendritic cells matured with the fiber knob domain and charged with GP33 peptide.

Interestingly, there is a correlation between the induction of GP33 peptide specific T cellular response and the number of dendritic cell stimulated by the fiber knob domain which were used for immunization.

More particularly, no response was detected after immunization with 3×10^4 knob domain-stimulated dendritic cells charged with GP33 peptide, although $CFSE^+$ cell rejection was observed.

The cytotoxic activity of spleen rat cells restimulated *in vitro*, was then tested, but no specific cytolytic activity was detected in mice immunized with 3 x 10^4 non stimulated dendritic cells (NS-DC). On the other hand, as illustrated in figure 7C, it has been demonstrated that effector cells obtained with fiber knob domain-stimulated dendritic cells were able to very efficiently lyse GP33 peptide-charged EL4 cells.

CONCLUSIONS

Results presented in the examples demonstrate that the effect of the fiber protein knob domain on dendritic cell maturation depends on the integrity and on the structure of the F β-sheet (amino acid residue at position 485 and 486 of the complete fiber protein), but not on other structures of the knob domain, such as the HI peptide loop. Moreover, the results in the examples demonstrate that the dendritic cell maturation activity induced by the fiber protein knob domain of the adenovirus does not require a trimeric structured knob domain.

Although these results are not presented in the examples hereabove, the applicant further demonstrated a dendritic cell-stimulated maturation activity with an adenoviral vector carrying chimeric fibers comprising a tail part and a shaft part of a serotype 5 adenovirus and of the serotype 3 adenovirus knob domain. These last results show that dendritic cell surface molecules are able to recognize adenovirus knob domains that are nevertheless phylogenetically distant from each other.

These results also demonstrate that interaction between the adenovirus knob domain and dendritic cell embryonic surface probably implies retained secondary peptide structures, rather than such individual amino acid residues retained in the knob domain.

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